BIOLOGICAL BULLETIN

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Marine Biological Laboratory

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BIOLOGICAL BULLETIN

INITIATION OF DEVELOPMENT IN NEREIS.

E. E. JUST.

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If any apology be needed for merely adding to the long list of eggs susceptible to agents of artificial parthenogenesis it may be suggested that initiation of development in annelids possesses some interest since annelid ova respond only with difficulty to agents that induce development.[1] We need but recall the case of *Chætopterus* (Loeb, '01*b*, Lillie '02, Allyn), of *Amphitrite* (Loeb, '01*a*, Scott), of *Podarke* (Treadwell), of the Pacific *Nereis* (Loeb, '13), of *Polynoe* (Loeb, '07, '08) and of *Nereis limbata* (Fischer, Lillie, '11, Loeb, '12*b*, '13)[2] to justify this statement. In all these eggs differentiation without cleavage is far easier to obtain than development closely simulating the normal. Among diverse agents few only will give cleavage in *Chætopterus* (Allyn, Loeb and Wasteneys). The case of *Thalassema* stands almost alone among annelids in giving development which is, according to Lefevre, to a surprising degree like the normal. Another instance among annelids of differentiation with cleavage artificially induced is worthy of note.

This report, however, on the initiation of development in *Nereis* by heat has, I think, special significance. The results here offered require an interpretation which concerns the fundamental theory of parthenogenesis and fertilization.

Experimental.

Certain preliminary experiments date from 1913. The experiments given here were performed during June, July, and

[1] Bullot claims to have produced normal development in *Ophelia* with artificial means.

[2] Loeb's experiments with *Nereis* ('12) were apparently incomplete.

August 1914, at the Marine Biological Laboratory, Woods Hole. The majority of the experiments deal with the effect of heat on the *Nereis* egg. Under *A* these experiments are described. Under *B* are described experiments with KCl.

A. The Effect of Warming on the Initiation of Development in Nereis.

Methods.—At first all sea-water used was heated, usually not beyond 75° C., to destroy any spermatozoa possibly present, cooled, and vigorously shaken before the experiment. But this is unnecessary, as my observations showed. I have kept *Nereis* eggs in sea-water during the cool days of June for thirty-six hours without even jelly secretion. During several seasons I have never found eggs spontaneously developing in sea-water, although eggs occasionally extrude part of their jelly. Moreover, in not a single uninseminated control in ordinary sea-water was a developing egg ever found. In many experiments in addition to the uninseminated control a batch of eggs from the same animal as those warmed was inseminated. It was thus clearly proved that the eggs subjected to warming are in no wise abnormal. For fear of contamination, the needless inseminated control was discarded in the later work.

For a given experiment the following procedure was adopted:

A small flask or a large test tube with a measured quantity of sea-water was placed in a large beaker of sea-water. This was warmed over an alcohol flame and the temperature kept constant by the use of thermometers in the flask and in the beaker. The eggs were generally from one female; if from several small ones, they were mixed so that the inseminated or uninseminated controls and the warmed eggs were always the same. The eggs in the initial experiments (see below) were either from females cut in the warm sea-water or they were put in the warm sea-water dry; *i. e.*, from a thoroughly dried female which was pricked to cause the escape of eggs. Eggs were also subjected to heat after washing by changing the sea-water several times during various intervals of time. By means of a capillary pipette measured quantities of eggs were transferred after exposure at varying intervals to five or to one hundred c.c. of ordinary sea-water. The

experiments were performed during the morning and afternoon following the evening that the worms were captured. A few experiments were performed during the evening of capture.

The point to be emphasized is that washing in sea-water so modifies the eggs that they do not respond readily, or at all, to parthenogenetic treatment.

The Experiments.

The experiments with heat may be divided into four groups as follows:

1. The initial experiments in which the eggs were cut from the animals while in the warm sea-water.
2. The experiments with dry eggs.
3. The experiments with eggs in warm "serum."
4. The experiments with washed eggs.

1. The Initial Experiments.

In the initial experiments, worms were cut in 5, 10, 25, 50, and 100 c.c. of sea-water at 30°, 32°, 33°, 34°, 35°, 36° C., the worms removed and the eggs exposed for from five to fifteen minutes.

The following experiments selected from a number give the details:

(*a*) July 22, 1:45 P.M. A female put in 100 c.c. of sea-water at 31° C. swims actively without discharging eggs. At 1:50, the temperature is 35° C. Eggs are cut out, the worm removed. Ten samples of eggs are removed to 5 c.c. of ordinary sea-water as follows: 2.03, 2:10, 2:25, 2:35, 2:45, 2:55, 3:05, 3:15, 3:25, 3:35 P.M.

At times the temperature rose to 36° C. and once to 36.5° C. Many eggs at the time of removal from the warm sea-water exhibited membranes standing off at an unusual distance, others were darker than normal, and a few had disintegrated. Later experiments showed that these changes are due to exposure at too high temperature. Even five minutes exposure at 37° C. will bring them about. The jelly is formed in the warm water, and often at 35° C. or above it is dissolved and disappears. This may be shown by examining eggs in India ink ground up in

sea-water. Many developing eggs are devoid of jelly hull, but the cortical changes are complete.

One hour and ten minutes after exposure, some eggs are in "blister" cleavages; that is, the protoplasm is irregularly budded. One hour and thirty minutes to two hours after the change to ordinary sea-water, among all gradations of cleavage-like patterns are some normal two and four-cell stages. The next day, Nos. 3 to 10 showed some real cleavages and a small per cent. of apparently normal swimming forms. Many are beaded or blistered, some are unsegmented "swimmers," and some two and four-cell swimming forms. Some eggs remain in the germinal vesicle stage.

(*b*) June 23, 11:00 A.M. A female placed in 50 c.c. of sea-water at 35.5° C. is rendered immobile but does not shed. Eggs are cut out at 11:02, the worm removed. Six samples of eggs are taken as follows: 11:18, 11:25, 11:34, 11:40, 11:45, 11:50.

Many eggs on removal from the warm sea-water show the jelly formed. The membranes after jelly formation are still a little farther from the eggs than in normal fertilization. Many eggs remain in the germinal vesicle stage with the cortex intact.

1:45 P.M. Fairly normal cleavages in Nos. 1 to 4.

June 24, 8:30 A.M. Swimming forms are found in the dishes. By far the best are those in Nos. 2 and 3.

The optimum time of exposure, therefore, lies between twenty-three and thirty-two minutes. Later experiments showed that the optimum exposure at 35° C. is at or near twenty-five minutes.

(*c*) June 24, several experiments were run at various temperatures. Those at 35° C. confirmed the findings of the previous ones. Temperatures ranging from 30° C. to 31° C. give no results; regardless of the length of exposure the eggs remain in the germinal vesicle stage.

The following experiment of June 24, at 33° C. is typical of a number of repetitions at this temperature:

(*d*) June 24, 11:15 A.M. A female placed in 25 c.c. of sea-water at 33° C. swims actively without discharging eggs. Eggs are cut out at 11:15 and samples taken at five-minute intervals up to 11:50. The temperature is practically constant. The samples taken are masses of eggs with the cortex wholly or (in

earlier ones) partially broken down. The cytoplasm is normal in color and the membranes normal.

1:35 P.M., many eggs are in cleavage.

4:00 P.M., many eggs are in late cleavage.

June 25, 9:00 A.M. The dishes show a good per cent. of very fine "swimmers." The cleavage seems almost normal.

Thirty-five minutes' exposure gives by far the highest percentage of swimming forms. As in all the experiments of this group, some eggs remain in the germinal vesicle stage with cortex intact.

If eggs be warmed in "egg-water" (sea-water charged by eggs that have remained in it for several hours) the results are no different.

To sum up, we find that eggs of *Nereis* cut out in warm sea-water and exposed to temperatures ranging from 33° to 35° C. develop with cleavage which is closely similar to the normal. Some eggs remain in the germinal vesicle stage. For the best percentage of swimming forms the optimum exposure at 35° is twenty-five minutes; at 33°, is thirty-five minutes.

2. Experiments with Dry Eggs.

Many of the experiments with dry eggs were run with the washed egg series. In the majority of cases eggs from one female thoroughly dried on clean filter paper were received in a dry watch glass.[1] These eggs were divided into two lots; one lot warmed in sea-water at the given temperature and the other washed by changing the sea-water several times, allowed to settle, and after draining placed in the warm sea-water.

A large number of experiments was made with dry eggs, in the attempt to determine the quantitative relations early found to control the number of eggs developing. Thus, with smaller quantities of warm sea-water every single egg quickly forms jelly and at least ninety-eight per cent. cleave, but with larger quantities of warm sea-water the percentages are lower.

As Miss Allyn found for *Chætopterus* cleavage appears to in-

[1] With dry eggs one must be careful for the mere drying will initiate changes as I have found. Eggs left on filter paper for from five to twenty minutes form jelly, a small per cent. cleave and a few swim.

terfere with the further development. I have never been able to get more than twenty per cent. of these eggs to reach the swimming stage. If one could determine definitely the quantitative relations this percentage might be increased. From the observations it appears that the optimum amount of warm water used varies; it depends upon the bulk of the eggs. While best results are got with small quantities of water, it is possible to use too little—three c.c. for instance, for the eggs for a large female. Jelly formation and cleavage are induced but swimming forms are less numerous than in the case of ten c.c. for about the same bulk of eggs.

The following are typical experiments of this group:

(*a*) July 16, 10:30 A.M. Eggs from a dry female in a dry watch glass are divided into two lots; one lot washed, the other transferred to 5 cc. of sea-water at 34° C. Samples out as follows: at 10:20, 10:50, and at five-minute intervals thereafter to 11:20.

2:00 P.M. Uninseminated control, no change. *Every single warmed egg had formed jelly: all have formed polar bodies.* At least half of these are in cleavage stages.

July 17. All dry eggs in some stage of cleavage, many of which are normal; some swimming forms in many of the dishes even after forty minutes' exposure.

(*b*) July 17, 9:58 A.M. Eggs from a dry female divided into two lots. Lot *A* in 5 c.c. of sea-water; Lot *B* in 20 c.c. of sea-water. Both exposed to 33° C. 10:01 jelly formation. Eight samples taken as follows: 10:05, 10:11, 10:16, 10:22, 10:27, 10:33, 10:38, and 10:43.

Lot *A* gave at least 95 per cent. of cleavage and a percentage of swimming forms in all dishes beginning with No. 3 (the 18-minute exposure). Lot *B* gave 75 per cent. of cleavage and best swimmers for 24, 29, and 35-minute exposures.

(*c*) Other experiments showed that the highest per cent. (100 per cent.) of jelly formation and of cleavage (98 to 99 per cent.) is in the smaller quantities of sea-water—5, 6, and 10 c.c. —whatever the temperature; a few swim. With larger quantities of sea-water at the various temperatures more eggs remain in the germinal vesicle stage. The lower exposures give most normal-looking swimming forms—trochophores scarcely to be

distinguished from the normal either while living or in sectioned material. The higher exposures give more abnormal swimming forms.

For comparisons I have selected the following tables from my notes to show the percentages of cleavage and of "swimmers" obtained with eggs from worms cut in warm sea-water and with dry eggs. It is apparent at once that while there is no appreciable difference in the percentages of swimming forms after warming either the "cut out" or the dry eggs, there is a marked difference in the percentages of cleaving eggs. This is the case in all the experiments.

July 12. Two females cut up at 9:55 A.M. in separate flasks of sea-water at 35° C. gave the following results:

Female No. 1.

	Sample Taken	Cleavage.	Swimming Forms.
No. 1	10:06	65%	1.5%
" 2	10:13	50	4
" 3	10:15	55	5
" 4	10:20	67	5
" 5	10:25	60	9
" 6	10:30	72	3
" 7	10:35	47	7
" 8	10:40	68	4—abnormal
" 9	10:45	62	very abnormal
" 10	10:50	33	very abnormal

Female No. 2.

	Sample Taken	Cleavage.	Swimming Forms.
No. 1	10:07	66%	3%
" 2	10:14	42	8
" 3	10:16	33	12
" 4	10:21	47	14
" 5	10:26	44	3
" 6	10:31	74	10
" 7	10:36	24	2—abnormal
" 8	10:41	81	5— "
" 9	10:46	32	very abnormal
" 10	10:51	17	very abnormal

July 20. Dry eggs in 30 cc. of warm water at 35° C. gave the following results:

Exposure.	Cleavage.	Swimming Forms.
18 minutes	90%	1%
23 "	"	15%
30 "	"	10%
35 "	"	7%

With dry eggs one may obtain 100 per cent. cleavage; with the eggs cut from worms in warm water one never gets more than 81 per cent. the average being very much lower as the figures given above show. With both kinds of eggs 20 per cent. swimming forms is the maximum, the optimum exposure for the various temperatures used being the same.

Experiments show that the use of warm "egg-water" does not improve the results.

3. The Experiments with Serum Eggs.

The effect of warming *Nereis* eggs in the body fluids was studied with difficulty mainly because of the scarcity of body fluid in *Nereis*. As Lillie has pointed out this worm is little more than a bag of eggs. The amount of blood present is negligible and unavailable for warming experiments. I therefore adopted the method used by Lillie—that of cutting up spent females. In his study this juice gave results comparable to the perivisceral fluids in *Arbacia*. For an experiment I minced as many spent females as I could get, using a small quantity of sea-water; the juice thus obtained is designated as "serum." While I think that my experiments with this serum are conclusive I wish to point out that *Nereis* is not the most favorable form with which to establish the fact of serum inhibition—certainly this is true for the method I used. It may be stated at the outset that as Lillie found for both *Nereis* and *Arbacia* I have found repeatedly that the "serum" of *Nereis* quite definitely inhibits fertilization. Furthermore, just as definitely does the serum inhibit initiation of development with warming. I cite experiments to give the details:

(*a*) July 15, 10:55. Eggs from one fine large female previously dried are divided into four lots. Eleven spent females are finely minced to procure twenty drops of "serum." Ten drops of the "serum" is added to each of two dishes containing 3 c.c. of sea-water; eggs added to both. One lot is warmed at 34.5° C.—Lot *A*; Lot *B* inseminated. Samples of *A* are taken at five-minute intervals up to 11:35. 2:00 P.M., 1 per cent. of cleavage in both lots. Next day no swimming forms in either. Eggs from the same female, Lot *C*, warmed in sea-water and Lot *D*, inseminated, develop.

During the afternoon of July 15 this experiment was repeated with the same results.

(*b*) July 22. Eggs warmed at 34° C. in serum plus sea-water (serum from the bodies of seven spent females cut up in two c.c. of sea-water): 10 drops plus three c.c. sea-water, 10 drops plus five c.c. sea-water and 10 drops plus ten c.c. sea-water.

Eggs exposed for twenty-five minutes. Less than one per cent. developed in any dish.

During August these results with serum eggs were verified. The highest per cent. of swimming forms obtained was one per cent.; this was with a very dilute serum. Not only do the eggs fail to cleave but fail in the great number of cases even to form jelly. In some cases the development of eggs inseminated in serum was farther advanced than the serum warmed eggs. Since in the case of the initial experiments the worms were cut up in sea-water, it may be that failure of a percentage of eggs to cleave is due to the inhibition of the escaping blood and tissue juice. With the dry eggs cut quickly on the dry watch glass this escaping juice cannot so easily contaminate the eggs.

4. The Experiments with Washed Eggs.

In *Platynereis* sea-water definitely destroys the fertilizing power of the egg. Even minute quantities of sea-water will render the egg incapable of cleavage although the spermatozoa may penetrate. Moreover, if the eggs of one female remain in a small quantity of sea-water, 5 c.c., for instance, for *thirty seconds* their fertilizing power is lost. And yet in nature, inseminated eggs begin to be laid in many cases *five or six seconds* after copulation (see Just, '14). In *Nereis*, therefore, it was thought that washings in sea-water by frequent changes through several hours might act as the sea-water does in such a surprisingly short time on *Platynereis* eggs.

During the June *Nereis* run, then, as many experiments as possible were conducted to determine the "fertilizable" period by inseminating at intervals eggs that had remained in sea-water with and without frequent washings. Lillie has shown for *Arbacia* eggs that the capacity for being fertilized decreased with the decreased secretion of fertilizin. He finds for *Nereis* also very much the same relationship.

Without going into details, it may be said at the outset that the egg of *Nereis* gradually loses its power of being fertilized and eventually reaches the condition of the *Platynereis* egg where insemination induces maturation only. I cite a single experiment.

June 28, 9:10 P.M. Dishes of eggs Nos. 1, 2, and 3 were set aside. The next day at 2:10 P.M. each dish of eggs was drained and divided into two lots—*A* and *B*. Lot *A* in each case was inseminated in the water which had stood over the eggs for seventeen hours. Lot *B* of each dish was inseminated in fresh seawater. No eggs in either lot of No. 1 developed beyond maturtion. In Lots *A* and *B* of Nos. 2 and 3, .1 per cent. or less went as far as the two-cell stage. Some eggs in all the dishes were in the germinal vesicle stage. No trochophores were found.

Eggs were frequently tied in bags of filter paper and placed in a beaker under running water for twelve hours. In other cases they were washed by changing the water at odd times during the day. It was found that eggs differ greatly with respect to the time that they must remain in sea-water before they lose their fertilizing power, but it may be clearly proved that washing or staling of *Nereis* eggs renders them incapable of being fertilized. This stage may be reached after three hours in seawater (cf. Just, '12).

This varying susceptibility proved very disappointing because I had suspected, not, of course, the degree of susceptibility present in *Platynereis*, but perhaps such as could be expressed more definitely.

Because of these results with washed and stale egg insemination, when the warm sea-water experiments were continued during the July *Nereis* "run" I was certainly unprepared for the results obtained. The following experiments are typical of a large number performed almost daily during the July and August "runs":

(*a*) July 11, 9:50 A.M. Eggs from one female divided into two lots; one lot put in sea-water. This lot transferred from the sea-water to warmed sea-water (35° C.). The eggs form jelly in the warm sea-water and make a mass which has to be shaken to obtain samples. 10:55, many have formed jelly and maturated, but most retain jelly with germinal vesicle intact. Some of

these eggs again subjected to heat; no results. July 12. Very few, 1 in 1000, swimming.

(*b*) July 15, 3:20 P.M. Eggs cut out and washed, put in 6 c.c. of sea-water warmed at 35° C. Samples taken at five-minute intervals for forty minutes. Next day: Majority are in germinal vesicle stage, at least seventy-five per cent. Less than one per cent. swimming.

(*c*) July 16, 9:35 A.M. Eggs washed ten times evening before and five times during this morning. Two series: *A* inseminated, *B* in warmed sea-water at 3:50 samples taken (ten in all) at five-minute intervals. Uninseminated control.

July 17, 1:30 P.M. No development in uninseminated control (few have cytolyzed). Inseminated eggs show that few have formed jelly (ten to fifteen per cent.). One per cent. have cleaved and some of these swim. Of the warmed eggs at least ninety-five per cent. are in the germinal vesicle stage with cortex intact. Less than one per cent. have formed polar bodies.

(*d*) July 16, 10:30 A.M. Eggs from a dried female divided in two lots; one lot washed in 100 c.c. of sea-water by changing the water four times. 10:40 A.M. In warmed sea-water, 34° C. Samples out at five-minute intervals for sixty minutes.

2:00 P.M. At least ninety per cent. in the germinal vesicle stage, small per cent. form jelly and divide. Next day, none swim.

I was tempted to discredit my June experiments after the first of these findings. I could only convince myself after running series after series of washed and dry eggs along with eggs cut out directly into warmed sea-water. Most workers in inseminating eggs obtain the sexual products in separate dishes, and add sperm. Such procedure succeeds admirably with *Nereis* giving one hundred per cent. of cleavage. But if eggs be cut out of *Nereis* in sea-water, divided in two lots, and washed once or twice, one lot being inseminated and the other warmed we get the surprising result that while every single inseminated egg develops, few of the warmed go beyond maturation. If the water over the eggs be changed a few times in ten minutes, ninety per cent. warmed in sea-water fail even to maturate.

This must mean that the egg of *Nereis* is so susceptible to

sea-water that warming fails after washing although fertilization is still possible. If fertilization be impossible (as in stale eggs) warming also produces no effect.

Washed or stale eggs warmed in sea-water charged by eggs that have remained in it for some time do not fare any better than those subjected to warmed sea-water; as in the first and second series of experiments this "egg water" makes no difference in the results.

I think that these facts are incontrovertible. Washing or even residence in sea-water for a short time interferes seriously with the effect of heat in initiating development.

Study of insemination of dry and washed eggs was made. Apparently there is a difference here of response to the spermatozoon. The dry egg is more irritable, jelly formation being extremely rapid. This is true of dry eggs inseminated in small quantities of sea-water. This behavior recalls that of *Platynereis*.

These results, moreover, might suggest that our methods are much too crude in the study of these extremely sensitive cells—the egg and the spermatozoon.

Summing up we may say concerning the effects of warming on the eggs of *Nereis*: (1) *That while eggs cut out of worms in the warm sea-water form jelly and divide in large numbers, a small per cent. swimming, some remain in the germinal vesicle stage.* (2) *That at least ninety-eight per cent. of the dry eggs form jelly almost all of which cleave: twenty per cent. become trochophores closely resembling the normal.* (3) *That eggs in "serum" fail to develop except in very small numbers.* (4) *That washed eggs even after but two or three washings develop if at all in small numbers.*

B. Effect of KCl in the Initiation of Development.

According to Fischer the eggs of *Nereis* after treatment with KCl will go through cleavage and produce trochophores. Lillie ('11), however, could not get the eggs after KCl treatment to go beyond maturation. During three seasons this had been my experience. This summer I studied the effect of KCl on washed and unwashed eggs.

If the eggs be washed two or three times before exposing to

the action of KCl every egg maturates but never more than one in a thousand swims. If the eggs be allowed to remain in sea-water from two to twelve hours with frequent changes of sea water the results are about the same. Dry eggs subjected to KCl treatment maturate, cleave once or twice, and produce, in one experiment at least, seven per cent. of swimming forms made up of unsegmented two and four-cell "swimmers."

The Experiments.

5, 10, 15, 20 and 25 per cent. 2.5M KCl were used. It was found that 15 per cent. 2.5M KCl in sea-water gave the best results. Typical experiments follow:

(*a*) August 12, 10:54 A.M. Lot *A*: Eggs from two females cut in 80 c.c. of 20 per cent. 2.5M KCl at 10:54. Lot *B*: Eggs from one dry female put in 3 c.c. of 20 per cent. 2.5M KCl. At 11:00 jelly formation in both. Samples of eggs taken from *A* and *B* as follows:

No. 1.....11:00
No. 2.....11:10
No. 3.....11:17
No. 4.....11:25
No. 5.....11:33
No. 6.....11:40
No. 7.....11:50
No. 8.....12:00

August 13, 12:00 M. Dry eggs of August 12 (Lots *A* and *B*). All maturated; some cleavage-like processes and some swimming forms after twenty minutes' exposure or more. Highest percentage (five) of swimmers after fifty minutes' exposure. These are unsegmented, two and four-cell swimmers.

(*b*) August 12, 12:05 P.M. Water changed three times on eggs during three hours and then placed in 20 c.c. of 20 per cent. 2.5 M KCl in sea-water. Samples taken at five-minute intervals up to 1:00 P.M.

August 13, 1:00 P.M. Washed eggs of August 12, all maturated; 1 in 1,000 swim.

Experiments during August 13 and 14 with fifteen per cent. 2.5 M KCl gave about the same results.

(*c*) August 15, 9:30 A.M. Two females quickly cut in 10 c.c.

of 15 per cent. 2.5 M KCl and removed. Samples taken as follows:

No. 1.....10:35
" 2.....10:50
" 3.....11:05
" 4.....11:20
" 5.....11:40
" 6.....12:00
" 7..... 1:25

These gave the next day swimming forms as follows:

No. 1.....4 per cent
" 2.....7 " "
" 3.....4 " "
" 4.....2 " "
" 5.....2 " "
" 6.....2 " "
" 7.....3 " "

(*d*) On August 17, eggs were washed by changing the water six times in five hours then subjected to 15 per cent. 2.5 M KCl in sea-water, samples being taken at sixty minutes and thereafter at five-minute intervals. One tenth per cent. (.1%) was the best result after sixty minutes in the KCl sea-water.

(*e*) *Combination of KCl with Heat.*[1]—An experiment of last summer was repeated except that dry eggs were used and the minimum exposure, five minutes, of the series the only one tried. The protocol follows:

August 17, 1:25 P.M. Dry eggs are put in 10 c.c. of 15 per cent. 2.5 M KCl in sea-water for five minutes; jelly formation almost at once. Eggs are then placed in 50 c.c. of sea-water at 35° C. Four lots removed to 100 c.c. of sea-water as follows:

No. 1.....1:55
" 2.....2:00
" 3.....2:05
" 4.....2:10

The next day, at 10:30 A.M., the percentages of swimming forms, largely unsegmented found were as follows:

No. 1.....(25 minutes' exposure to heat)..... 3 per cent.
" 2.....(30 " " " ")..... 7 " "
" 3.....(35 " " " ")..... 27 " "
" 4.....(40 " " " ")..... 25 " "

[1] It will be recalled that Allyn used a combination treatment of KCl and heat on the egg of *Chætopterus* with rather different results from those mentioned here with *Nereis*. Her method however, was different.

It appears, therefore, that with KCl, and KCl and heat, washed and unwashed eggs alike will maturate, but that the dry eggs alone respond with cleavage or the production of swimmers.

Discussion.

In the egg of *Nereis* Lillie discovered a substance, *fertilizin*, which has the property of agglutinating *Nereis* sperm. This substance may be detected in the water in which the eggs have remained for a short time. If, however, the eggs be washed by changing the water two or three times the fertilizin is no longer secreted in detectable quantities, *i. e.*, there is not enough to agglutinate the sperm. Such eggs are none the less fertilizable by sperm, giving off at the time of insemination more fertilizin, all of which is then utilized or completely thrown off during the cortical changes. It therefore follows that at the time of shedding the egg is laden with free fertilizin ready for secretion. This conclusion is supported by additional facts. In the first place I have pointed out above that the dry egg or egg in small quantities of sea-water is hyper-irritable—that is, if jelly formation may be taken as index. If one inseminates the eggs of *Nereis* dry or in small quantities of sea-water the jelly formation is extremely rapid. Jelly formation is correspondingly slow in washed and stale eggs. The breeding behavior noted night after night for several seasons is significant: freshly shed eggs at the surface of the sea excite numbers of males to shed their sperm around the shedding or recently spent female. Lillie's experiments (Lillie and Just) on this sperm shedding reflex, moreover, prove that the egg loses fertilizin once in the sea-water. The "dry" and "washed" eggs of my experiments, then, are physiologically different: the dry egg has all its available fertilizin content, the washed egg has secreted part of this substance.

Lillie has shown that the eggs of *Nereis* will not fertilize in the tissue juices of the animal; my experiments show also that the body juice of spent females inhibits fertilization. Unlike the washed egg, the "serum" eggs possess fertilizin but its action is inhibited.

But it is on the basis of experiments on *Arbacia* that Lillie has developed the fertilizin theory as an explanation of the me-

chanism of fertilization. Without going into details it may be said that in *Arbacia* it is found that the egg secretes a substance, fertilizin, whose presence is capable of quantitative determination and which is necessary for fertilization since first, eggs washed free of it are no longer capable for fertilization; second, fertilized eggs no longer secrete it; and third, eggs after membrane formation with butyric acid are not capable of fertilization and do not give off the substance. The perivisceral fluid of *Arbacia*, moreover, produces an inhibiting effect on fertilization preventing the action of fertilizin on the egg.

My results with warming *Nereis* eggs parallel to a striking degree these facts brought out in the studies of fertilization in *Nereis* and *Arbacia* (Lillie, '12, '13*a*, '13*b*, '14). Eggs washed free of the bulk of fertilizin will not develop however long the warming treatment lasts; serum inhibits the artificial initiation of developmental processes; only the dry eggs with their full content of fertilizin when suddenly shocked with elevation of temperature respond with jelly formation and cleavage. It would seem, therefore, as I have suggested for *Platynereis*, that fertilizin is just as essential for artificial initiation as for normal fertilization. The difference seems to be that for artificial initiation more fertilizin is required. Further attempts at Woods Hole this summer to induce artificial parthenogenesis in *Platynereis* strengthen this belief; a percentage of *Platynereis* eggs will fertilize in small quantities of sea-water; the same bulk of eggs in the same amount of water fail to respond when subjected to warming.

If, therefore, as Loeb ('12*a*) says, "fertilization is primarily and essentially artificial parthenogenesis"; or if "a theory of fertilization must also be a theory of parthenogenesis at least for the phenomena common to both"; and if "similarly a theory of fertilization must be consistent with the facts of parthenogenesis" as Lillie ('14) suggests; these experiments, we are forced to conclude, make another link in the chain of evidence which supports the theory that fertilization is essentially a process of the egg. The spermatozoon initiates the development of the egg, as does warming, through the activation and the binding of the fertilizin.

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SPERM AGGLUTINATION AND FERTILIZATION.

FRANK R. LILLIE.

In a recent paper on "Cluster Formation of Spermatozoa Caused by Specific Substances From Eggs" Loeb ('14) has presented a criticism of my theory of fertilization (Lillie '13*b* and '14), based on observation of the California sea-urchin *Strongylocentrotus purpuratus*. My own observations were made on *Arbacia punctulata* of Massachusetts, and it would appear that part at least of Loeb's criticism was due to certain differences in the two forms, for he has now stated (Loeb, 1914*b*, p. 318, footnote) that the "cluster formation" of the spermatozoa may find its explanation "on the assumption of an agglutination at least in the case of *Arbacia*," as I maintained; it is therefore not a "tropistic reaction" as he thought probable from his observations in California. This was one of the chief differences of opinion. A second one was in regard to the source and significance of the substance in the fluid of egg suspensions that caused such agglutination; Loeb maintained that it was merely the dissolved chorion (*i. e.*, jelly layer) of the egg, and that after this was removed the eggs no longer produced the agglutinating substance, and yet were capable of fertilization; whereas my contention was that the agglutinating substance was a secretion of the egg soaked up by the jelly, as by a sponge; that the eggs produced it for a certain length of time after the removal of the jelly, and lost their power of fertilization after they ceased to produce it.

These criticisms cut at the foundation of my theory. Inasmuch as the correction of the tropistic interpretation of agglutination is given only in a footnote to another paper, and no correction of the source of the agglutinating substance has yet appeared, it is incumbent on me to consider the criticisms carefully; at the same time I wish to take the opportunity to explain certain points that appear to be open to misinterpretation, and to record some new observations.

1. "Cluster-formation" versus Agglutination.

The phenomena exhibited by sperm suspensions of *Arbacia* with which we have to deal are of four distinct types, which it is essential to distinguish sharply: (1) activation; (2) aggregation; (3) agglutination, (4) mass-coagulation.[1] (1) That the activity of spermatozoa is affected by substances in the sea-water requires no argument. The subject is discussed in study V (Lillie, 1913*a*, pp. 519–532). (2) Aggregation of spermatozoa may be brought about by tropistic reactions. In my paper on the "Behavior of Spermatozoa," I devoted a great deal of attention to such aggregation phenomena and the distinction from phenomena of agglutination (1913, pp. 532–548 and pp. 551–552). Among other things I pointed out that aggregation as a tropistic phenomenon implies a gradient,[2] and that the spermatozoa never adhere, however crowded they may be; there is no observable physical change of the spermatozoa and the slightest agitation suffices to disperse them again. Such tropistic phenomena may be exhibited in response to CO_2 and other acids (*Nereis*), or certain constituents of egg secretions, to mention only chemotaxis.

(3) Agglutination of spermatozoa on the other hand requires no gradient, and the spermatozoa adhere physically to such an extent that the agglutinated masses may be preserved intact in killing fluids; its degree is a function of the concentration of the agglutinating medium, and is also different in different species. Agglutination is non-toxic, not limiting the life of the spermatozoa; it is reversible, its duration depending on the concentration of the agglutinating medium; it cannot be repeated if the reaction is complete, at least within the time limits of my experiments, even though the spermatozoa remain motile;[3] finally motility of the spermatozoa is a prerequisite to a decided reac-

[1] The reaction here referred to is a lethal phenomenon. It possibly involves cytolysis with subsequent adhesion of the spermatozoa.

[2] It is important to notice that the spermatozoa of suspensions may produce gradients through their own activities. Thus I pointed out that autogenous aggregation reactions in sperm suspensions of *Nereis* arise from the positive chemotaxis of the spermatozoa to their excreted CO_2, giving rise to very striking phenomena (Lillie, 1913*a*, pp. 519–521 and pp. 538–540). It is conceivable that such a tropistic phenomena is involved as a part factor in the agglutination phenomena under discussion.

[3] Glaser (1914) also comes to this conclusion.

tion; evidently because the physical change on which the reaction depends is not sufficient to cause adhesion except when the spermatozoa positively collide.[1] These six criteria definitely define the phenomenon.

Agglutination is positively distinct from aggregation. It is an entirely different biological phenomenon. The two may, however, be exhibited simultaneously, as when a drop of egg secretion of *Arbacia* is injected into a sperm suspension of the species. In such a case the spermatozoa exhibit positive chemotaxis to one constituent of the egg secretion, and are agglutinated by another (the fertilizin). The separateness of these two substances was maintained in my first publication on the subject and demonstrated by repeated experiments (see Lillie, 1913*a*, p. 549, and 1914, pp. 545–546).

(4) The phenomenon of mass-coagulation is, on the other hand, a lethal irreversible phenomenon. It may be exhibited in response to various agents, such as KOH, NaOH, salts of lanthanum and cerium,[2] etc., and in some cases the secretions of the eggs of other species or their blood. Hitherto I have not adequately defined this phenomenon as distinct from the agglutination phenomena, though in my last study (1914), I noted the distinction (p. 541). The phenomenon is essentially lethal, but not all destructive agents exhibit it; thus acids, so far as I have observed, destroy the spermatozoa without causing mass coagulation. The phenomenon is irreversible, and this suffices to distinguish it from true agglutination, even if no other criterion were available. However, it exhibits quite a different aspect from agglutination; in the latter the sperm masses tend to take on a spherical form; if originally elongated they contract into balls or break up into smaller masses which become spherical, thus offering considerable resemblance to a phenomenon of surface tension, as Loeb notes. The peripheral spermatozoa are in violent movement until the time of reversal. In the mass-coagulation reaction, on the other hand, there is no such surface tension effect, strands anastomose

[1] Loeb argues that the necessity of movement on the part of the spermatozoa for the appearance of this phenomenon removes it from the category of true agglutination; but this seems to me to be a purely arbitrary criterion.

[2] My attention was called to the action of the salts of these metals by a letter from James Gray of Cambridge University.

with other strands and form a net-work and the movements of the spermatozoa soon cease.

The substances of egg secretions, which I have hitherto called hetero-agglutinins, belong to this category, in some cases at any rate. Though I will not assert that there is no such substance as a hetero-agglutinin in the real sense of agglutination, yet the substance in *Arbacia* blood, or egg secretions, the effects of which on *Nereis* sperm I have previously studied, should be regarded as a toxic rather than an agglutinating substance, having the mass coagulant action. As I stated in my last paper, p. 541, it produces a permanent coagulum in *Nereis* sperm suspensions; "in this respect the action differs from the iso-agglutination, which is without toxic effects."

We must keep firmly in mind the distinctions between aggregation (tropisms) agglutination, and mass-coagulation. Agglutination, with which we are particularly concerned, is distinguished from aggregation by the facts that it occurs in the absence of a gradient, it involves physical adhesion, and cannot be repeated if the reaction is once complete; it also is characterized by a high degree of specificity.[1] From mass coagulation it is distinguished by the facts (1) that it is non-toxic, (2) reversible, (3) dependent on motility of the spermatozoa. Agglutination occurs so far as I have observed with certainty only in response to egg-secretions of the same species.

For description of the phenomena of agglutination of sperm by egg-extractives of the same species, I must refer to my previous paper (Lillie, 1913*a*); the phenomenon in *Arbacia* is a true agglutination in the sense defined, not a tropistic reaction, nor yet a mass coagulation. Loeb has admitted this for *Arbacia*, and I would therefore venture to suggest the probability that the phenomenon which Loeb has described in *Strongylocentrotus* and termed "cluster formation," which he interprets with some reserve as a possible tropistic reaction, is also true agglutination, which differs only quantitatively from *Arbacia* and *Nereis*. The

[1] Loeb admits that the "cluster formation" exhibits a high degree of specificity. It is therefore inconsistent to interpret the reaction, as he also does, as a "possible tropistic phenomenon" because such phenomena so far as we know do not exhibit specificities of this kind. Agglutination phenomena, on the other hand, as is well known, commonly exhibit equal specificity of a similar kind.

conditions under which it occurs, in response to egg secretions of the same species, its character, reversibility, and the specificity of the reaction are identical with *Arbacia*. It is apparently, however, less pronounced, and therefore not so readily recognizable of itself as an agglutination phenomenon. Even the "apparent surface tension phenomena" which Loeb describes for the clusters—"Short streaks or cylinders contract into spherical masses, the above described clusters; and long cylinders break up into a series of small clusters"—are the same as I previously described for *Arbacia* (1913*a*, pp. 550–551).

Loeb's interpretation of the "cluster-formation" as a possible tropistic reaction confuses the two sets of phenomena—viz., aggregation (a true tropistic phenomenon) and agglutination—which sperm suspensions may exhibit to the egg-sea-water of its own species. But the aggregation (tropism) can take place only when there is a gradient from the secretion to the spermatozoa. This is realized under the conditions of my experiment of injecting a drop of egg-sea-water into a fresh sperm suspension beneath a raised cover slip; in such a case the two phenomena take place simultaneously viz.; aggregation in the form of a ring around or in the introduced drop (depending on concentration), and agglutination. These two phenomena are produced by two constituents of the egg-sea-water, as I have already maintained.

For the study of the aggregation phenomena therefore it is desirable to employ an agent which has no agglutinative action. This I did in an extensive series of experiments by the method just referred to (1913, p. 533 ff.). To illustrate:—a drop of a $^1/_{100}$ dilution of a saturated solution of CO_2 in sea-water injected into a sperm suspension of *Nereis* in sea-water mounted beneath a raised cover-slip is marked within a few seconds by the formation of a ring of active spermatozoa within the margin of the introduced drop, and separated from the general sperm suspension by a clear zone nearly free of spermatozoa 1.5 to 2 mm. in diameter. I interpreted the ring formation as a positive reaction to the attractive substance (CO_2 and acids generally); the spermatozoa follow the gradient from the suspension into the drop containing CO_2 a certain distance, *i. e.*, up to a certain concentration, and

are there arrested. The proof of this interpretation is found in the fact that, if increasing concentrations of CO_2 are used, the ring forms outside the drop and becomes progressively wider, *i. e.*, the migration ceases at a distance from the center which increases with CO_2 or acid concentration (see 1913*a*, pp. 536–538). Loeb suggests that the ring formation with a clear external zone around it is "an indication that the spermatozoa are negatively chemotropic to the strong egg-sea-water, and possibly positively chemotropic to the more diluted egg-sea-water, or to the dense collection of spermatozoa in the ring." The latter suggestion is of course untenable as a primary cause, for the ring-formation is precisely the phenomenon to be explained. It is also unnecessary to assume any negative tropism; the ring formation is due to a limitation of the positive movement by concentration. This is fully discussed in the paper referred to above, but Loeb does not allude to the discussion.

2. The Source of the Agglutinating Substance.

Professor Loeb has also taken issue with me on the question of the origin of the agglutinating substance. He regards his experiments as proving that the substance which causes the "cluster formation" is not formed in the egg but in the chorion; *i. e.*, in the layer of jelly which surrounds the egg. On the other hand I regarded it (and still hold to the opinion) as a secretion of the egg; with which the jelly of course becomes saturated.[1] Loeb's observations again were on *Strongylocentrotus* and mine on *Arbacia*. The issue is a real one even though the chorion is itself a secretion of the egg in earlier stages.

Loeb's conclusion was based on his observation that if the chorion be dissolved off in dilute hydrochloric acid in sea-water, the naked eggs transferred to sea-water produce no detectable amounts of the agglutinating substance any more, whereas the acid sea-water contains it in large quantities. My conclusions were based on the observation that when eggs of *Arbacia* are deprived of jelly (chorion) by shaking, or a prolonged series of

[1] Glaser (1914) also agrees substantially with me: "the agglutinating substance is located in greatest abundance in the jelly and the eggs also contain this material," p. 371.

washings, they still continue to produce the agglutinating substance in sea-water, though in much diminished quantity; in my full paper, which Loeb had not the opportunity of consulting, I gave series of measurements on this point (1914, pp. 532–538); I also pointed out that in immature ovaries containing many primary ovocytes, but some mature eggs, the quantity of agglutinating substance produced was relatively very small (1914, p. 530), and I therefore suggested that the substance was secreted by the eggs at the time of maturation and was soaked up by the jelly as by a sponge. The eggs, however, continue to produce it after maturation, as I shall show. The immature eggs have as thick a chorion as the mature eggs; therefore the agglutinating substance cannot be merely dissolved chorion. I recognized the possibility of the view expressed by Loeb, investigated it as fully as possible at the time, and rejected it.

Since Loeb's paper has appeared, I have repeated his experiments and found my former observations and conclusions confirmed in all respects:

Experiments.—The optimum concentration of HCl for removal of jelly without injury to eggs was found to be 50 c.c. sea-water + 1.4 c.c. N/10 HCl. 1.2 c.c. N/10 HCl in 50 c.c. sea-water did not fully remove the jelly, and 1.6 c.c. caused too much injury to the eggs evidenced by heavy agglutination and later cytolysis. In an experiment of July 17, 1914, the three above concentrations were used. The complete removal of the chorion in the intermediate concentration was demonstrated by observation of the eggs in a thick suspension of India ink in sea-water; even the minutest traces of adherent jelly can readily be detected by this method, but it was all gone. The eggs were then washed as follows: 10.11 A.M. 42/6 c.c.; 10.40 51/5 c.c.; 10.58 50/4 c.c. The supernatant fluid was then tested and found to be free from sperm agglutinating substance; thus furnishing proof that all originally contained in the jelly had been washed out. At 11.20 the supernatant fluid was poured off leaving only 5 c.c. in the tube. The eggs were allowed to settle, and at 11.25 the supernatant fluid was tested and gave a 9–10-second agglutination reaction with fresh sperm suspension. Thus these eggs entirely deprived of jelly by HCl are producing agglutinating substance.

At 4.25 P.M. the eggs were washed again 5/0.7 c.c. and the new fluid gave a 14-second reaction. The next morning the same eggs were washed again 5.5/1 c.c. The new fluid gave a 6–7-second reaction.

These results may be expressed in a different way: thus in an experiment of July 20, a series of eight successive washings of eggs deprived of jelly by acid sea-water represented a dilution of the agglutinating substance contained in the acid sea-water remaining with the eggs of 12,700,800 times. But the acid solvent itself was negative at 1/800 dilution: it was of 400 agglutinating power. In other words, after the removal of the jelly the eggs themselves had produced a sufficient quantity of the agglutinating substance to account for the tremendous difference; and they were still producing it.

These eggs without jelly are fertilizable, as Loeb states, but only 37 per cent. segmented in a heavy insemination of the first day in the experiment of July 17, and only a small part of these developed to the ciliated stage, none of which were normal, most being stereoblastulae and incapable of farther development. The result is entirely similar to that described in my last paper (study VI, '14) for the fertilization of eggs deprived of jelly by shaking and subsequent washing.

The same experiment was repeated on July 18, 20 and 21, with identical results: the eggs from which jelly is entirely removed by HCl continue to produce the sperm-agglutinating substance (fertilizin) so long as they live, but their capacity for development after fertilization is much reduced.

In all experiments at least three concentrations of acid were used, and in each experiment it was observed that when the concentration was sufficient to dissolve the jelly there was a good deal of agglutination of the eggs, and in the later washings a great many eggs broke down liberating their pigment. As I have previously shown, broken-down eggs liberate a substance (anti-fertilizin) which neutralizes the sperm agglutinating action of the fertilizin. Therefore, when a sufficient percentage of the eggs are breaking down, the production of sperm-agglutinating substance (fertilizin) by intact eggs may be entirely masked.

I have no intention of disputing Professor Loeb's observations

for *Strongylocentrotus*. But they merely prove either that *Strongylocentrotus* sperm is not so delicate an indicator as *Arbacia* sperm, or that the method employed by Loeb was inadequate to detect small quantities of fertilizin. In *Arbacia* the eggs continue to charge the sea-water with sperm-agglutinating substance after complete removal of the jelly, whether by shaking and repeated washings, or by HCl; and the substance continues to be formed as long as the eggs remain fertilizable and living, no matter how often the eggs are washed. *The eggs of Arbacia secrete the substance as I previously maintained. It is not merely the "dissolved chorion."*

It might possibly be objected to this conclusion that the continued appearance of the agglutinating substance in egg suspensions in sea-water after removal of the chorion indicated merely previous adsorption of the substance of the chorion. But the indefinite continuance of its production is inconsistent with the idea of a mere secondary removal of an adsorbed substance. The idea is also inconsistent with the fact that *Nereis* eggs have no jelly at the time laying, but produce a similar sperm agglutinating substance. In this form the jelly also is secreted by the egg after insemination.

Finally if it can be shown that the jelly of immature eggs is entirely devoid of the sperm agglutinating substance, my position that this substance is a later secretion of the egg is rigorously proved. As noted above I maintained the probability of this view in my previous paper (Study VI). This summer my first experiments were undertaken to investigate this point anew.

Fortunately the season was late, and not a single *Arbacia* was ripe when I began work (June 8). This applied to males as well as females: so it was impossible at first to secure ripe sperm as indicator. I therefore made extracts of immature ovaries to be kept for subsequent testing from three females (1, 2, and 3, June 8). June 11 extracts of ovaries in sea-water were made from females 4, 5, and 6: numbers 4 and 6 contained only ovocytes; No. 5 had a large number of ripe ova in addition. On June 16 extracts 1–6 were tested with *Arbacia* sperm suspension: 1, 2, 3, 4, and 6 were absolutely negative; no agglutination. No. 5 gave a strong agglutination reaction lasting about one minute.

It is highly improbable that the agglutinating substance had been destroyed in five of the six, and retained in the only one (No. 5) of the extracts which was made from ovaries containing some ripe ova. So far as these observations go, the jelly of immature ovocytes is free of agglutinating substance.

Again on June 15 I made extracts from ovaries of three females in two of which ripe ova were practically absent, the third had a few. Tested the same day the two former extracts had no sperm agglutinating properties; the third gave slight agglutination.

The females appeared to mature slightly earlier than the males, so that for these experiments I was forced to use rather thin sperm suspensions (mixed more or less with immature spermatozoa), which were probably not as delicate indicators as one could wish. However the difference between the ovaries containing ripe ova and those without was perfectly distinct. Later when fully ripe males could be had all ovaries contained ripe ova.

The following observation also tends in the same direction: June 27, 1914—Three females were selected, of which number 1 was the ripest attainable, the eggs flowing freely out of detached ovaries, and very few ovocytes occur; numbers 2 and 3 were the least mature attainable; number 2 had very few detachable ova, mostly late ovocytes with a sprinkling of ripe eggs; number 3 had quite a few detachable ova with a large proportion of ripe eggs. The ovaries of all three were cut up equally, and sea-water added to each to make 10 c.c. When the ova and ovaries had settled they stood at 1.5 c.c. in 1, at 1.3 c.c. in 2, and 1.5 c.c. in 3. After five hours, tests of the agglutinating strength of the supernatant fluids were made with clear fresh sperm.

No. 1 gave a 10-second reaction at 1/800 dilution.

No. 2 gave a 6-second reaction at 1/10 dilution.

No. 3 gave a 7-second reaction at 1/40 dilution.

Thus No. 1 is 80 times the strength of 2 and 20 times the strength of 3. In general the fertilizin production is proportional to the ripeness of the ovaries.

There is not the slightest doubt in my mind about the demonstrative character of these observations. The appearance of agglutinating substance in the jelly of *Arbacia* eggs is secondary,

and takes place probably at the time of breaking down of the germinal vesicle.

Loeb's contention that the agglutinating substance is merely dissolved chorion therefore does not hold for *Arbacia*. With this his argument against my fertilizin theory also falls: "Moreover if it should turn out that the substance which is responsible for the cluster formation is identical with the substance which Lillie calls "fertilizin," which is very likely the case, Lillie's theory becomes untenable, since this substance does not, in all probability, originate from the egg, but from the chorion and since there is, as we have seen, no connection between the presence of this substance and the power of the eggs of being fertilized" (pp. 136–137—Loeb, '14).

In this statement Loeb sums up the essentials of his criticism; since I have shown that "cluster formation" is true agglutination (which Loeb now admits), and that the agglutinating substance (my fertilizin) is not dissolved chorion but a true secretion of the eggs which continues to be produced after the chorion is removed, the entire stated criticism becomes ineffective. There *is* a connection between the presence of this substance and the power of the eggs of being fertilized: the substance can first be demonstrated at the time that the power of being fertilized first arises, viz., after breakdown of the germinal vesicle; it can be demonstrated as long as eggs retain the power of being fertilized, whether the chorion be removed or not, and it disappears absolutely after fertilization, as I showed in my previous paper (study VI, p. 553, 1914).

3. Other Criticisms.

Another objection raised by Loeb is that "the supernatant sea-water of the eggs of *Strongylocentrotus franciscanus* will not induce cluster formation of the sperm of *Strongylocentrotus purpuratus:* yet the latter sperm fertilizes the eggs of *franciscanus*," from which he argues that the fertilizin of *Strongylocentrotus franciscanus* can not be necessary for the fertilization of its eggs. An error in logic is involved here; agglutination of sperm is merely an indicator of the presence of a certain substance, which is none the less present in *franciscanus* even if *purpuratus*

sperm does not reveal it; it *may* nevertheless be activated by *purpuratus* sperm and this is the essential point in the theory.[1] Agglutination of sperm is of no significance except as indicator. As I pointed out in my previous paper, binding of the fertilizin by sperm receptors, *i. e.*, the chemical reaction, is a thing entirely distinct from agglutination; if such binding causes a certain kind of physical surface change of the spermatozoa of suspensions of a certain minimum concentration, they agglutinate; otherwise not. Agglutination is a valuable indicator that enables us to make certain analyses, and that is all. The same principle of fertilization may hold in the entire absence of sperm agglutination.

Another objection in which Loeb supports the possibility of superposing fertilization on parthenogenesis will be dealt with in a separate paper. My contention in this case is that the possibility of such superposition always rests upon incompleteness of the parthenogenetic reaction; if the fertilization reaction be complete, whether by parthenogenesis or insemination, it cannot be repeated. Everybody admits that eggs fertilized by sperm cannot be refertilized; it is a logical impossibility that eggs "fertilized" by parthenogenetic reagents should be refertilized. The problem of the apparent contradiction involved in Loeb's and Herbst's contention of superposition works out in the manner indicated. A study of this problem by one of my students will appear soon.

Loeb cites as a farther difficulty of my fertilizin theory, which he says I have not considered, "that in addition to the membrane forming substance still another, namely a correcting agency, is necessary for causation of the development of the egg." Though

[1] Loeb states (1914, p. 135): "If the phenomenon of cluster formation were inseparably associated with the power of the eggs of being fertilized, we should expect that sperm should only be able to fertilize the eggs of a species if the egg-sea-water of the same species caused the cluster formation of the sperm." I have never maintained that agglutination ("cluster formation") is inseparably associated with the power of the eggs of being fertilized, but merely that a certain substance produced by the egg is a necessary factor in fertilization. In some cases this substance (fertilizin) produces agglutination of the sperm of its own species, and this reaction furnishes an indicator of its amount, when present, or of its absence. In other cases such an indicator is lacking: I do not find that supernatant sea-water of the eggs of the starfish (*Asteria forbesii*), for instance, agglutinates its own sperm; but I have evidence, to be published elsewhere, that the mechanism of fertilization may be explained in the same way as in *Arbacia*.

I cannot accept this statement of the problem, I have nevertheless taken into consideration the fundamental fact, to which Loeb alludes, in the full account of my experiments, which appeared after Loeb's paper was in press. The fundamental fact is simply that the fertilization process in some cases can be divided in two sharply marked stages. This is perhaps most simply and convincingly shown by my own experiment (Lillie, 1911) of removing the spermatozoon from the egg of *Nereis* after it had already induced the cortical changes, with the result that the developmental phenomena came to a standstill before the first cleavage. I cannot agree with Loeb that the second stage involves a factor corrective of an excess action of the factor of the first stage. I think it is probable that we have a progressive process readily capable of resolution into two stages.

In my complete paper (Lillie, 1914) I considered the second phase of fertilization with reference to the new theory, and may refer the reader to the discussion there given (study VI, pp. 582–584). Here it is only necessary to point out that the "fertilizin" theory is at least as well adapted to account for the two stages as the "lysin" theory.

4. Conclusion.

I may be allowed to emphasize the essential features of my theory with some added light thrown by the work of this summer. The fundamental conception is that all agencies initiating development of the egg do so by the same means, viz., activation of an ovogenous substance, which I have termed fertilizin. This conception brings fertilization and parthenogenesis under one conception. I further assumed that such activation in the case of fertilization was caused by union of a constituent substance of the spermatozoon (the sperm receptors) with the fertilizin, and that the activation expressed itself by consequent union of the fertilizin with certain egg substances (the egg receptors). The reaction was thus conceived in terms of the Ehrlich side-chain theory, and was represented diagrammatically accordingly.

That certain chemical combinations form an essential feature of the fertilization reaction cannot be open to doubt. I have not previously taken into account the consideration that the

occurence of such reactions, taking place, as they must, across the egg membrane, is dependent on physical conditions of the membrane, especially its permeability to the substances concerned. In speaking, as I did, of five blocks to the fertilization reaction, I was concerned only with the chemical reactions involved. There may be other blocks of a physical nature. Indeed these were much in evidence in the fertilization of *Asterias*, which I studied in the first part of the summer, and shall report on elsewhere. Another important consideration is that the reaction must also be dependent on environmental conditions such as temperature, ionic constitution of the medium (see Loeb, '14*b*), etc. Blocking of fertilization may also arise from such causes.

Continuing the exposition of the theory; I identified the fertilizin of *Arbacia* with the substance found in the fluid of egg suspensions which causes agglutination of sperm suspensions of the same species. This phenomenon cannot possibly be lacking in significance, for it furnishes direct evidence of a combination of egg and sperm derivatives; the phenomenon itself is not concerned in fertilization, for a single spermatozoon may fertilize an egg. Neither does the absence of such agglutination in other species affect in the least the conclusion that may be drawn from *Arbacia:* because we may have a combination of egg and sperm derivatives without any sperm agglutination. The agglutination is incidental, the combination is the essential thing.

The fertilizin theory in its essential aspects is not dependent on the identification of fertilizin and sperm agglutinating substance. I believe in their identity; but if it were proved, as Loeb has sought unsuccessfully to do, that the agglutinating substance is not essential for fertilization, the fertilizin theory would still not be attacked in its essence. The conception that initiation of development is essentially a phenomenon of activation would still stand in opposition to theories of external agents acting directly by corrosion (cytolysis), or coagulation, or what not. The egg could still be regarded as a self-contained system with no more than the usual environmental relations. It is only from this point of view that the complex phenomena of parthenogenesis and fertilization can be united in a logical whole.

The theory of the identity of fertilizin and sperm agglutinating substance rests upon a considerable body of ascertained facts (see study VI), and it gives us at once a point of attack and a working hypothesis of considerable value. I have been able to show for instance that the origin of the capacity of the egg for being fertilized can be understood on this basis; that the cessation of fertilization capacity can also be so understood; and that the physiological sterility (prevention of polyspermy) of fertilized eggs is readily explained by the neutralization of the fertilizin by a substance (anti-fertilizin) demonstrably present in the egg.

On the other hand the theory does not postulate that the fertilizin of all forms should agglutinate sperm of its own species. There may be many forms in which the union of the sperm receptors with fertilizin does not produce such physical changes of the spermatozoa as to lead to agglutination. In those cases in which agglutination does occur we have a reaction very useful in analysis; but it cannot be too strongly emphasized that the agglutination itself is to be regarded merely as an indicator of the essential reaction.

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OBSERVATIONS ON THE SPERMATOGENESIS OF THE GALL-FLY, DRYOPHANTA ERINACEI (MAYR).

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INTRODUCTION.

Dryophanta erinacei is one of the gall-producing *Hymenoptera* having two generations in the year: males and females in the spring, and females alone in the fall. The fertilized eggs of the bisexual generation produce females exclusively, while the unfertilized eggs of the female generation produce both males and females.

The material for this study was obtained on April 13, 1914, from galls occurring on white oak trees (*Quercus alba*) in the neighborhood of Cincinnati. The galls are smooth spherical protuberances on the bud-scales, several millimeters in diameter. Usually one, but occasionally two, and sometimes three galls very close together are found at the end of a single twig. Twigs bearing galls were cut off, brought into the laboratory, and the cut ends inserted in sand moistened with water. Ten days later males and females emerged from the galls, and continued emerging for two weeks. Copulation took place immediately after emergence.

These galls supplied all stages of developing males and females from the late larva to the imago. For fixation Petrunkewitsch's fluid was used; penetration being facilitated by making a longitudinal incision through the body wall. In some cases ovaries and testes were dissected out, but better results were obtained when the organs were left in situ and the entire animal sectioned.[1]

Embedding was done in rubber-paraffin, and serial sections cut 10 μ in thickness. Two methods of staining were used: safranin and light-green, and iron-haematoxylin with or without counterstain. After dehydration the stained sections were cleared and

[1] For assistance in collecting and preserving material I wish to acknowledge my indebtedness to Dr. Annette F. Braun.

mounted in euparal. Euparal offers several advantages over balsam as a mounting medium. Thus its use obviates running stained sections through absolute alcohol; since sections may be transferred directly from 95 per cent. alcohol to euparal. Next the index of refraction of euparal is low 1.483. And lastly euparal dries quickly, so that sections may be studied at the end of twelve to twenty-four hours after mounting, without danger of injury.

Observations.

The testes of the late larva and early pupa show primary spermatocytes at the end of the growth period as large polygonal cells having a reticulated nucleus containing a poorly defined nucleolus, often of a bipartite character (Fig. 1). The nucleolus does not take the safranin stain as deeply as the chromosomes, and thus differs markedly from the chromosome nucleolus of the primary spermatocytes of many *Hemiptera*.

A true primary spermatocyte division does not occur. Instead, a small mass of cytoplasm free of chromatin is constricted off, forming the so-called polar body. Preparation for this suppressed or abortive division begins with a change in the outline of the cell, the spermatocyte assuming a pear shape (Figs. 2, 3 and 4). From the narrow end of the cell and forming the stem of the pear, extends a short filar process. At the base of this process, which at first glance suggests the tail of a spermatozoon, is often found a light basic-staining spherule which may or may not be a centrosome. While these changes are taking place in the cytoplasm the nucleus undergoes a slight contraction and the chromatin passes through a series of transformations terminating in the formation of chromosomes (Fig. 4).

The next step in the process is somewhat uncertain and there may be some question as to seriation. It seems that after the chromosomes are completely formed, they become massed in clumps at one side of the nucleus, and from these masses distinct loops extend toward the opposite side of the nucleus (Figs. 5 and 6). The cell shortens, the filar process becomes less distinct (Fig. 6), and a portion of the cytoplasm is constricted off (Figs. 7 and 8). As this is taking place the nuclear membrane appears very irregular in outline but seems to remain intact. Inside the

nuclear area the chromosomes are in the form of single rods whose free ends extend toward the polar body. There is every appearance to indicate a resistance of the part of the chromosomes against a tension pulling toward the polar body. Distinct spindle fibers are not to be seen, but the cytoplasm contains a reticular structure which may represent a poorly developed spindle. The polar body is quickly cut off from the cell to which, however, it may remain attached for a considerable length of time (Fig. 15). The free polar body of Fig. 8 belongs to a cell in an adjacent section. Polar bodies cut in various planes are frequently seen in the spaces between spermatocytes at this time (Figs. 8 and 16) and throughout the second spermatocyte division. The complete absence of polar bodies in cysts containing cells with the chromosomes in the looped condition of Figs. 5 and 6 makes it almost certain that the looped stage precedes that of Figs. 7 and 8, in which the chromosomes show free ends.

Preparations for the second spermatocyte division follow very rapidly. After the formation of the polar body, the second spermatocyte rounds up; the knot of chromosomes separates into distinct, short, thick, curved rods, 12 in number (Fig. 9). In the cell figured here, a late prophase, the nuclear membrane is fairly distinct. Details of spindle formation were not observed. Figs. 10 and 11 show characteristic side-views of spindles at metaphase. The chromosomes seldom lie in one plane so that counting even in polar views is a difficult matter. In such views, as in Figs. 12 and 13, 12 chromosomes can be counted with considerable accuracy in the majority of cases.

A characteristic late telophase is shown in Fig. 14 which resembles to a striking degree a somatic mitosis, and strongly suggests that the chromosomes have been divided longitudinally. In later stages of this division (Fig. 15) the chromosomes become packed into dense compact masses, so that it is impossible to determine the number of constituent chromosomes in the daughter groups. When reconstitution of the nuclei occurs (Fig. 16), these masses break up into slightly bent rods of ragged outline. In cross section these rods appear as dots of which 12 can often be counted. Counts of the daughter groups of chromosomes made in this way are not very satisfactory, since one is

never sure that a cross-section includes all of the rods or that a single rod has not been cut more than once.

The spermatids formed by this division seem therefore to be equal in size and chromatin content, and all of them develop into spermatozoa. There is no evidence of a heterochromosome or chromatoid body passing undivided into one of the spermatids.

By the end of the second spermatocyte division all of the polar bodies are detached and show signs of disintegration, fragments being frequently seen in the intercellular spaces giving the appearance shown in Fig. 16.

The relatively distinct outline of the chromosomes seen in this last figure persists for but a short time and is completely lost in the young spermatids. Figs. 17 and 18 are early stages in the transformation of the spermatids into spermatozoa.

Such in brief is an outline of the main features of development of the germ cells in the male of *Dryophanta* from the growth period to the spermatids. There is but one true maturation division—that of the second spermatocyte. The first spermatocyte division is indicated by the pinching off of a small quantity of chromatin-free cytoplasm which forms the so-called polar body.

Discussion.

Doncaster in his studies of the gametogenesis of the gall-fly, *Neuroterus lenticularis*, arrived at certain conclusions which may be considered at this point. This species of *Hymenoptera* has a similar life-history to that of *Dryophanta*. Thus according to Doncaster the female generation emerges in April from galls formed during the preceding summer and immediately lays eggs in oak buds (species?). Early in summer the galls appear from which males and females emerge. After copulation the female lays eggs in the tissues of young leaves at the side of a small vein. From the galls resulting, females emerge in the following spring.

As in *Dryophanta*, therefore, the fertilized eggs of the bisexual generation develop into females; while the unfertilized eggs of the female generation produce both males and females.

Doncaster found that the first spermatocyte division is abortive—a small portion of the cytoplasm being constricted off as the polar body. This is followed by a resting stage which resembles

the metaphase of a true division, but is distinguished from it by the persisting nuclear membrane and the position of the chromosomes at one end of the nucleus near the broad end of the cell. No nuclear division takes place but the nucleus becomes oval in shape and the chromosomes generally contract to form a compact mass lying across its center. In some cells at least this chromatin mass seems to divide—one half passing to each side of the oval nucleus. The chromatin may finally disperse and give rise to a condition resembling the first spermatocyte in which the chromatin has begun to appear. "Possibly the division of the chromatin inside the nucleus, which occasionally seems to occur, is the persistent remnant of a true nuclear division, or it may be compared with the 'intranuclear karyokinesis' described by Kostanecki in the parthenogenetic eggs of *Mactra*" (p. 93). Toward the end of the rest stage the chromatin becomes grouped in the form of large elongate granules or small bands having a more or less meridional arrangement under the membrane.

The second spermatocyte division in *Neuroterus* is a true mitotic division in which the haploid number of chromosomes, 10, appears on the spindle to be equally divided between the daughter cells. There is also a small stained body lying outside of the spindle which passes undivided to one of the spermatids.

In the spermatogonia and in mitotic figures of nerve cells in the developing nervous system Doncaster finds the halpoid number of chromosomes, 10, but in mitoses of immigrant mesoderm cells the diploid number, 20.

The eggs layed by the females of the bisexual generation undergo two maturation divisions; leaving 10 chromosomes for the female pronucleus. The spermatozoon brings into the egg 10 chromosomes, and 20 chromosomes appear on the cleavage spindles. The parthenogenetic eggs of the female generation may be divided into two groups: Those which undergo maturation and develop into males; those which omit the maturation divisions and develop into females. In the first group 10 chromosomes are found in the cleavage divisions; in the second group 20. Since any female produces only one kind of egg, there are male-producing females and female-producing females.

Mitoses in the nervous system of all females show the diploid number of chromosomes.

Returning now to *Dryophanta* I should like first to consider the stage represented in Figs. 5 and 6, which I believe corresponds to the second spermatocyte resting stage mentioned by Doncaster in *Neuroterus*. The figures at first glance suggest the synapsis stage of other insects, but in view of other facts it is difficult to interpret the condition as a fusion of chromosomes. Earlier stages such as the prophase shown in Fig. 4 display the same number of chromosomes as appears in the second spermatocyte division, 12, which is assumed to be the haploid number approximately. Since there is no evidence in *Dryophanta* of an intra-nuclear division of these 12 chromosomes into two groups, a true synapsis at this time would be equivalent to a second "reduction." A more probable interpretation of this "looped stage" and one that is warranted by a close study of the sections is that the limbs of a loop are the halves of a chromosome that has undergone a temporary and incomplete splitting. With the next step in the process, the formation of the polar body, the split disappears and the chromosomes have every appearance of being single, solid rods (Figs. 7 and 8). The latter condition might of course be brought about by breaking of the loops at the middle, but in that event one would expect to find twice as many single chromosomes as loops. Such is not the case, for the number of unsplit chromosomes is the same as the number of loops so far as could be determined. Reversing the seriation at this point would of course change the interpretation offered here; but the main reason for placing the looped stage before the other, as has been mentioned above, is that there is no evidence of polar body formation at this time. And to this may be added the fact that the outline of the cell at the looped stage as shown in Fig. 6 represents an intermediate condition between that of Fig. 4 in which there can be no question about polar bodies being absent, and Figs. 7 and 8, in which the polar bodies certainly are present.

An actual resting stage, if one occurs at this time, must be of very short duration. The second spermatocyte division follows very quickly after the formation of the polar body. Fig. 9 represents a prophase of this division in which the chromosomes are surrounded by an intact nuclear membrane. The spindle

area of the second spermatocyte is rather distinctly marked off from the rest of the cytoplasm (Fig. 10) and suggests that the nuclear membrane disappears very slowly.

Polar views of the metaphase display, as nearly as could be determined, 12 chromosomes, presumably the haploid number. It would seem that each chromosome is divided quantitatively by a longitudinal splitting; although it must be remembered that attempts at verifying this conclusion by studying the constituents of the daughter groups are not satisfactory owing to the tangled condition of the chromosomes.

I find nothing resembling the small stained body which in *Neuroterus* according to Doncaster passes undivided to one of the spermatids. As Wilson has observed this body is of the same nature as the chromatoid body seen in the growth-period and spermatocyte-division of *Pentatoma*. The chromatoid body is of rounded form, dense and homogeneous consistency, and after double staining with haematoxylin or safranin and light green is at every stage colored intensely blue-black or brilliant red, precisely like the chromosomes of the division period or the chromosome-nucleoli of the growth period. Nevertheless Wilson finds that the body is neither a chromosome nor any kind of a chromosome and takes no visible part in the formation of the spermatozoa. In the transformation of the spermatids it wanders far into the sperm-tail and is at last cast off altogether.

I have not yet had opportunity to study the maturation phenomena of the egg in either generation of *Dryophanta*, but observations confined to individuals of the bisexual generation point to general conclusions which differ somewhat from Doncaster's views regarding the chromosomal relations in the alternate generations. In the material at my disposal spermatogonial divisions are not abundant enough to determine the number of chromsomes. While mitoses abound in the somatic cells of male larvae and pupae, it is difficult to find good clear metaphases; but wherever counts were possible, the number found was 12 (Fig. 19). In the follicle cells of the ovary I have found it less difficult to count the chromosomes. Figs. 20, 21 and 22 are drawings of metaphase plates of such cells in which the numbers are respectively 13, 14 and 13.

In the somatic cells of both males and females one occasionally finds mitotic figures containing a much larger number of chromosomes, but such cases are in the nature of exceptions and no one would contend that they represent an average condition. If there is such a thing as constancy in the number of chromosomes in the majority of somatic cells, the constant is in the neighborhood of 12 in both males and females of the bisexual generation. Because this is the number of chromosomes found in the second spermatocyte division, 12 is assumed to be the approximate haploid number. Now in any case where an accurate count is difficult or impossible in the somatic cells, it is always possible to determine with certainty that the number is very much less than the expected diploid number 24. In view of the fact, that in the honey-bee it is said that the somatic mitoses show a very much higher number of chromosomes than occurs in the gonial cells, somatic mitoses should not be used as a safe and reliable method of determining the diploid number. There may however be some significance in the fact that a large number of somatic cells of both males and females of *Dryophanta* contain a number of chromosomes that approximates the number found in the dividing spermatocyte rather than a multiple of this number.

Any definite statement regarding the origin and significance of this condition must await examination of the maturation and cleavage spindles of the egg. However, the facts at hand do suggest that the males and females of the bisexual generation of *Dryophanta* develop from eggs whose chromosomes have undergone reduction in maturation. The slightly large number of chromosomes found in the females somatic tissues may or may not be of significance, but if sex determination has its basis in the chromosomes, a difference in the method of distribution of the chromosomes in maturation may explain why some of these eggs develop parthenogenetically into females and others into males.

In a recent paper Nachsheim has summed up in a general statement the results of investigations dealing with sex-determination in *Hymenoptera* as follows: "Die Männchen der Hymenopteren entstehen aus unbefruchtete Eiern, die zwei

Richtungskörper abgeschnürt und eine Reduktion ihrer Chromosomenzahl erfahren haben. Sie besitzen also nur ein Chromosomensortiment, das mütterliche, und infolgedessen muss in der Spermatogenese die Reduktionsteilung unterbleiben. Die Weibchen der Hymenopteren besitzen beide Chromosomensortimente, also die diploide Chromosomenzahl in ihren somatischen Zellen, da sie aus befruchteten Eiern ihre Entstehung nehmen oder—bei den Blatt- und Gallwespen—zwar ebenfalls aus unbefruchteten Eiern, aber aus solchen, die den Reifungsteilungen ihre Chromosomenzahl nicht reduciert haben; entweder findet in diesen Eiern überhaupt nur eine Reifungsteilung statt, oder beide Reifungsteilungen sind Aquationsteilungen. Der zweite Richtungskörper kann also. . . . an Stelle der Spermatozoons treten, d.h. der zweite Richtungskörper bringt in Verbindung mit der Eikern dasselbe Geschlecht hervor wie der Eikern in Verbindung mit einem Spermakern" (pp. 220–221).

My findings in the somatic chromosomes of *Dryophanta* raises the question as to whether females of the bisexual generation are produced parthenogenetically from eggs that do not undergo reduction in maturation. An examination of maturation stages in the egg is necessary to decide this point and material for this purpose is being collected at the present time.

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EXPLANATION OF PLATES.

The figures are camera drawings made at table level with Zeiss apochromatic objective, 1.5 mm. and compensating ocular, 12. There has been some reduction in reproduction.

PLATE I.

FIG. 1. Primary spermatocyte at the end of the growth period. Male pupa.

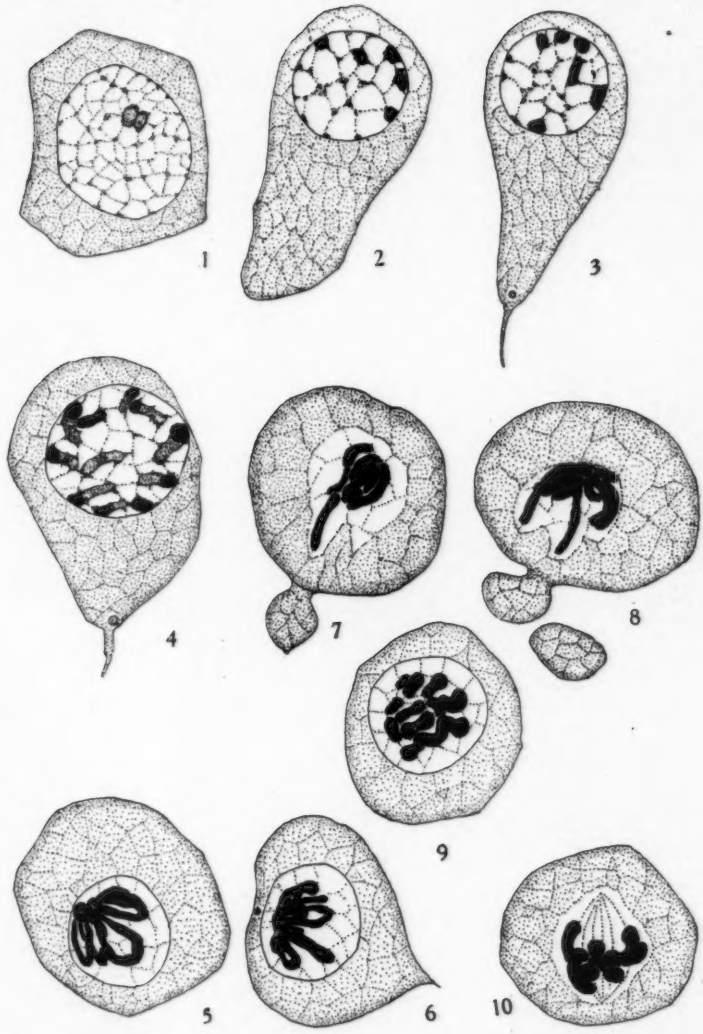
FIGS. 2, 3 AND 4. Primary spermatocytes undergoing changes in outline preliminary to the formation of the polar body.

FIGS. 5 AND 6. Primary spermatocytes having chromosomes in the form of loops or split rods.

FIGS. 7 AND 8. Stages in the cutting off of the polar body. Fig. 8 contains a second polar body belonging to a cell in a neighboring section.

FIG. 9. Prophase of the second spermatocyte division showing 12 chromosomes.

FIG. 10. Side view of the second spermatocyte spindle at metaphase.



H. L. WIEMAN.

PLATE II.

FIG. 11. Side view of the second spermatocyte spindle at metaphase.

FIGS. 12 AND 13. Polar views of the second spermatocyte spindle at metaphase showing 12 chromosomes.

FIG. 14. Second spermatocyte spindle at late anaphase showing a free polar body near the upper end of the cell.

FIG. 15. Second spermatocyte at telophase with a polar body attached to the upper daughter cell.

FIG. 16. Early spermatid, reconstruction of the nuclei. Polar body fragments near the upper cell.

FIGS. 17 AND 18. Stage in the transformation of spermatids into spermatozoa.

FIG. 19. Metaphase chromosome group in the mitosis of a developing wing, showing 12 chromosomes. Young male pupa.

FIGS. 20, 21 AND 22. Metaphase chromosome groups of ovarian follicle cells, showing 13, 14 and 13 chromosomes respectively. Late female larva.

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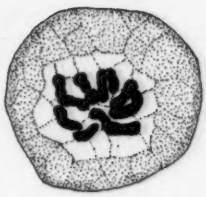

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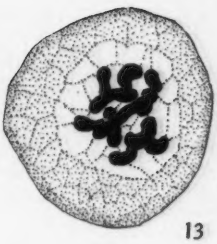

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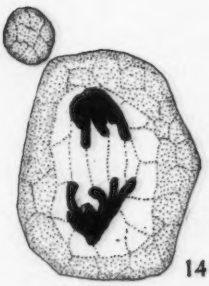

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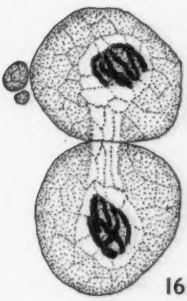

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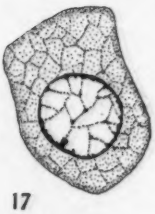

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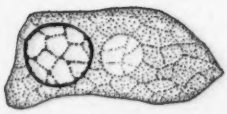

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EGG ALBUMEN AS A CULTURE MEDIUM FOR CHICK TISSUE.

OLIVE SWEZY.

Egg albumen as a culture medium for chick tissue *in vitro* has received but scant attention from experimentalists, in spite of the fact that it forms the natural medium, in part at least, of the embryo chick. In a recent series of experiments, however, results have been obtained which show that all the usual manifestations of cell activity, noted by various observers in other culture media, were to be met with in cultures made from egg albumen, and have, I believe, demonstrated satisfactorily its entire adaptability to that use. These experiments were carried on in the laboratory of Prof. S. J. Holmes, to whom my thanks are due for his kindness in giving advice and assistance throughout the course of the work.

The technique followed has been that outlined by Burrows and Carrel, modified to suit the different conditions under which the work had to be carried on, using embryos varying in age from twenty-four hours to fourteen days. Of these it was found that the most successful results were obtained from embryos of from ten to fourteen days growth, though all showed considerable activity. Fragments of all the organs of the body, including the brain and spinal cord, were used, but the most active growths were obtained from the heart. Several series of preparations were made by cutting up the entire embryo into minute particles in a small amount of Ringer's solution and egg albumen, stirring and shaking these rapidly for a few minutes and then placing a small drop of the mixture on the slide and sealing in the usual way. By this process cultures could be made containing but a few or even single cells. The medium used has been egg albumen alone or mixed with varying proportions of egg yolk, Ringer's solution and extract of muscle tissue. Egg yolk proved entirely unsatisfactory because of the impossibility of seeing what was taking place within it. The best results were obtained from egg

albumen alone and with mixtures of albumen and muscle tissue extract, the latter being prepared from embryo chick tissue and added to the albumen either before or after making the culture. Egg albumen coagulates to a more or less firm consistency and thus gives one of the conditions apparently requisite for the growth and activity of the tissue cells.

Owing to the viscosity of the albumen, considerable care is necessary in handling the specimens when it becomes needful to transfer the culture to a fresh medium, the usual method of procedure being to cut away the old albumen with a sharp knife. When, as is frequently the case, the outgrowth seemed to be mainly on the surface of the glass, and thus could not be transferred in the usual way without the loss of the greater part of the growth, another method was used. Inverting the cover glass the albumen was removed with forceps and pipette, several changes of Ringer's solution successively placed over the culture and, after removal of this, a fresh drop of albumen was added to the culture and it was again sealed up.

The latent period, before the beginning of activity of the culture, lasted from half an hour to several days. Usually, in good preparation, active amœboid movements began within half an hour after being put on the slide. At that time along the border of the tissue could be seen the elongated, outpushing cells forming a fringe along what was before a clear cut outline, with a few scattered cells lying at some little distance from the main mass. These cells displayed very active amœboid movements that are less common in the older cultures though still present to some extent. When these cells are chilled or disturbed they contract and become rounded. On a number of cultures groups of cells showed long clear processes extending outward, sometimes branched, with the ends breaking up into short filaments. These were in all cases cultures which included portions of the brain or spinal cord from a four-day chick. An attempt was made to photograph one of these cultures but the length of time necessary was sufficient to chill the slide and, on examination, it was found that the processes had all been retracted. Subsequent incubation had no effect on the culture, though disintegration did not take place for several days. In all the cultures

these processes disappeared, were retracted apparently, in the course of fifty to seventy hours and no further evidences of them were seen. In the preparations made by shaking up the finely cut embryo with Ringer's solution, a greater or less number of single cells were found. In the course of a few days these were greatly increased in number with a distinct massing together of the cells, usually along the outer border of the drop of albumen. Owing to accidents of various kinds these were not carried along far enough to show the tissue formation noted by Carrel.

The most marked instance of tissue formation was that apparent in a culture made from the heart of a fourteen day chick, which, at the end of twenty days was encircled by a new formation five times the diameter of the original piece of tissue. This new formation was several cells in thickness and composed of fusiform and polygonal cells, sometimes massed together, forming a network, or in other places showing distinct cell boundaries. Among these cells many showed division figures at various stages. Around the outer margin of the mass of cells and extending nearly three-fourths of the entire distance around it, the cells had taken on a different character. Here they had become flat, thin and elongated in a direction parallel with the margin of the circle. This formation was several cells in thickness with the cells closely matted together and forming a distinct boundary that was conspicuous without the aid of a lens. The remaining one-fourth of the margin was occupied by cells actively pushing outward.

To test the effects of cold on the growth of the tissues, the embryo was sealed up in a stender dish containing Ringer's solution and placed in the ice box of the refrigerator with the temperature but a few degrees above zero, Centigrade. The first of these was used the second day and behaved like normal tissue. Most of those kept in the refrigerator for a number of days became infected with bacteria. The longest period of cold storage which gave successful cultures was four days, from January 31 to February 4. One half hour after making the cultures from this embryo the cells were moving out in an active condition in four out of the sixteen cultures made. The subsequent history of these cultures was the same as that of unrefrigerated tissue.

The longest period during which tissues have been kept alive

without any evidences of necrobiosis has been ninety-three days, and in the majority of these cases death has been caused by infection with bacteria or molds or other accidents, and, not, apparently, by any lack of vigor in the tissues themselves. This, in general, seems to be true of most of the cultures which appear to be in a thriving condition after the second day or third day, and especially where renewals of the culture medium have been frequent, and precautions have been taken to avoid tearing or otherwise injuring the tissues. However disintegration frequently takes place from no apparent cause.

Egg albumen presents some difficulties when a stained preparation from the culture is desired, on account of its avidity for stains. In the first stained preparations made it was impossible to distinguish the outlines of the cells, and the study of the specimen seemed a hopeless task. This difficulty was later overcome by the following methods: the cover glass was inverted and placed on the mouth of a vial containing a quantity of osmic acid. The mouth of the vial was small enough to be completely covered by the cover glass and yet not touch the preparation. After fixing in this manner for ten minutes the cover glass was placed in a stender dish containing distilled water and left for a number of hours. Frequent agitation and changes of the water removes the greater part of the albumen, leaving the tissue adhering to the glass, which may then be put through the alcohols and stained in the usual way. With this method very clear preparations may be obtained.

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THE INFLUENCE OF PRODUCTS OF PATHOLOGIC METABOLISM ON THE DEVELOPING TELEOST OVUM.

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In his recent work on pathological human ova, after careful sifting of anatomical evidence, Mall[1] arrives at the conclusion that the failure of large numbers of ova to develop normally is to be traced to diseases of the uterus. According to his view, which is supported by obstetrical and gynecological data, diseases of the uterus are the primary cause of the faulty implantation of the ovum. This in turn makes proper nutrition of the developing embryo impossible thus leading to various degrees of malformations by arresting development. The deformed embryo is eventually aborted after it has exhausted its inadequate means of subsistence in the uterus. Full-term monsters would be born from such deformed embryos if they were not hindered in their further development by starvation. According to this theory, therefore, an apparently healthy ovum discharged into a diseased uterus fails to develop normally owing to its defective implantation.

Mall studied largely pathological ova of the first two months and the interpretation of the numerous cases described by him seems justified. Practically all pathological ova of the early months studied by him as well as by other investigators, exhibited the condition of faulty implantation, so that it is not unwarranted to regard this condition as the direct cause of monstrous development.

A consideration of some instances of arrested, defective or even monstrous development found after full-term birth would suggest, however, that there must be also some other factors

[1] Mall, F. P., "A Study of the Causes Underlying the Origin of Human Monsters." *Journ. of Morphology*, Vol. XIX., 1908; "The Pathology of the Human Ovum" in Keibel-Mall "Handbook of Human Embryology," 1910.

which primarily interfere with normal development. Such defects as rudimentary development of one or both eyes, congenital absence of both arms, hydrocephalus, possibly also cases of congenital deafness, to mention only a few that are well known to occur, can, in the writer's opinion, hardly be traced to defective implantation. The results of investigations in experimental teratology by Panum,[1] Dareste[2] and more recently by Stockard[3] and Bardeen[4] would seem to suggest that some physico-chemical factors may be at work in a great number of cases of pathological development. These factors may in some instances be the primary cause of terata, while in other cases they may be only secondary contributing causes.

The experimental teratologists subjected developing ova in very early stages to changes in the physico-chemical nature of the environment and found that various monstrosities could be produced under these conditions. It was impossible, however, for them to control the results of experimentation, as they could not predict the type of monster which would result from the employment of the same factors. The experiments of Stockard, where a more or less definite monstrosity—cyclopia or monophthalmia—appeared with considerable certainty in a large percentage of embryos developing in magnesium chloride or alcohol solutions, mark a distinct progress in this field of inquiry, because they paved the way towards experimental control of monstrosities occurring in nature.

To the writer Stockard's work suggested the possibility that the monstrosities met with in higher animals and man may to a certain extent be due to the influence of injurious substances found in the circulation under pathological conditions. While this hypothesis could not be applied to bacterial toxins on account of insufficient knowledge, it seemed that some substances thrown into the circulation in various metabolic diseases may be re-

[1] Panum, "Entstehung der Missbildungen," 1860.

[2] Dareste, "Recherches sur la production de monstrosites," Paris, 1891.

[3] Stockard, C. R., "The Artificial Production of a Single Median Cyclopean Eye in the Fish Embryo by Means of Seawater Solutions of Magnesium Chlorid," *Arch. f. Entwmech.*, Vol. XXII., 1907; "The Influence of Alcohol and Other Anæsthetics on Embryonic Development," *Am. Jour. of Anat.*, Vol. X., 1910.

[4] Bardeen, C. R., *Jour. of Experimental Zool.*, 1907; *Am. Jour. of Anat.*, Vol. XI.

sponsible for pathological development. Thus the etiology of defective or monstrous development would be traced to the pathological metabolism of the mother or possibly even of the father. For, as Bardeen[1] has shown, a normal, healthy ovum of the toad, if fertilized with sperm which had been injured by exposure to the action of X-rays, will give rise to a deformed embryo.

With this idea in mind the writer conducted during the summer of 1914 experiments on eggs of *Fundulus heteroclitus*. The eggs of this fish are easily obtained at Woods Hole and are excellent material for experimentation. The investigations on the fish eggs are of a preliminary character, and were undertaken to ascertain the influence of some toxic substances occurring in pathological metabolism on the developing egg.

The number of these substances being rather large while the spawning season is limited to a few weeks, it was impossible to try more than a few of the chemicals. Urea, butyric acid, lactic acid, sodium glycocholate, acetone and ammonium hydroxide were tried as to their effect on the development of fertilized eggs. Definite results were so far obtained only with butyric acid and acetone.

Ten c.c. of a 1/12–1/14 molecular solution in 50 c.c. of sea water was found to give the greatest number of monsters when butyric acid was used. The eggs were submitted to the action of this solution for 20 hours after they had reached the eight-cell or sixteen-cell stage, *i. e.*, 3 to 3½ hours after fertilization. While under this procedure numerous monstrosities were at first obtained, the method failed almost completely in later experiments. I therefore employed developing eggs in the first stages of division (2- and 4-cell stages) when many monstrosities were produced even after a sojourn of thirty hours in the butyric acid solution. But it seems to me that the reason why the method failed with the eggs in more advanced cleavage stages was that the time of exposure was too long, as very many eggs were dead by the end of that treatment, and that with an exposure of 10 or 15 hours better results would have been obtained.

There is, however, as important difference in the effect which

[1] Bardeen, C. R., *Jour. of Experimental Zool.*, 1907; *Am. Jour. of Anat.*, Vol. XI.

this toxic substance has upon developing eggs in the first and second or in the third and fourth divisions. In the former case anterior hemiembryos, dwarf embryos with deformities of the eyes or of the otic vesicle, and malformations of the most extreme kind were predominant, while in the latter deformities of the eye such as cyclopia and monophthalmia, etc., were mostly observed. In either case, however, there were very few embryos in which only the nervous system was affected. In most of the deformed embryos all organ systems were more or less involved in the malformation.

Similar results were obtained with acetone in sea water, varying in concentration from 20–50 c.c. of a molecular solution in 50 c.c. of sea water. In this mixture the eggs remained from 24–72 hours from the eight-cell or sixteen-cell stage. In every case great numbers[1] of monsters similar to those already mentioned were produced.

The monstrosities in both series of experiments with butyric acid and acetone being essentially alike it will not be necessary to describe separately the deformities produced by each.

Cyclopia and asymmetric monophthalmia were found to occur rather abundantly. There were also some cases of asymmetric monopththalmia in which an open orbit was found on the side lacking the eye. It is of some interest to note in this connection that the eyeless orbit in such cases is usually closed on the outside by periorbital tissues. The anatomy of the head of such embryos may probably reveal some interesting conditions. Other cases of asymmetric monophthalmia were found in which an apparently free eye had developed on the yolk-sac at a considerable distance from the embryo. Probably the most striking of the results obtained in this investigation were some eggs in which nothing could be observed but an eye. In only one case this eye seemed to be perfectly developed, while the other solitary eyes had "coloboma"-defects, the fissure of the chorioid still being patent. Only a few (five or six) of these malformations are recorded, but in spite of their rare occurrence they are very significant from the standpoint of experimental embryology. At the

[1] No attempt was made to ascertain the percentage of the deformities found in these experiments, this part of the work being deferred to later investigation.

present time it is, obviously, impossible to account for the occurrence of these remarkable cases. However, it is hoped that an anatomical investigation of early stages in the development of eggs subjected to the influence of the environmental modifications used in these experiments, may give at least a clue as to what may have happened in the development of these eggs. Practically all other known deformities of the eye such as total blindness, or presence of lenses only, or presence of supernumerary lenses were frequently found.

To the student of the physiology of development the occurrence in these experiments of large numbers of anterior hemiembryos which seem to be closely analogous to those obtained by mechanical means by Roux,[1] Endres,[2] Morgan[3] and K. Ziegler[4] will be of special interest. As will be pointed out soon the formation of the hemiembryos in these experiments may also possibly be due to similar factors.

A great number of embryos were hydrocephalic and so far as could be determined it is reasonable to expect that an anatomical investigation may reveal in some deformed embryos oedematous conditions, also herniae and other mechanical obstructions which played a part in their formation.

Striking abnormalities of the heart and blood-vascular system were found in all malformed embryos with the exception of those which showed only median cyclopia. Some were entirely devoid of the heart, while other possessed an exceedingly delicate tube in its place which was practically straight and of about the size of the intestinal blood vessels in a normal embryo of a corresponding stage. The rate of the heart beat varies with the degree of the abnormality of the organ, and is, as a rule, very slow in all monstrous embryos. The range of variation in the development of the blood vessels is very wide. There may be merely blood islands scattered on the yolk-sac, rudimentary, imperfectly connected, or in some instances more or less normal vessels.

[1] Roux, W., "Gesammelte Abhandlungen zur Entwicklungsmechanik der Organismen," II., 1895.

[2] Endres, H., "Anstichversuche an Froscheiern," *Sitzber. d. zool.-bot. Sektion d. schlesischen Ges. f. vaterländische Kultur*, 1894.

[3] Morgan, T. H., "The Formation of the Embryo of the Frog," *Anat. Anz.*, 1894.

[4] Ziegler, K., "Zur Postgenerationsfrage," *Anatomische Hefte*, Vol. LXVI., 1902.

Twins were found only in a few cases and only once were true "Siamese" twins observed. They were much deformed, had one common heart and only vestigial eyes. Several eggs were recorded, in which an anterior duplicity had developed. In one of these latter cases the components of the duplicity were totally blind, hydrocephalic, their hearts were very delicate, the blood vessels rudimentary and the yolk-sac was covered with dense networks of richly pigmented blood islands.

These monstrous embryos hatch only very rarely, most of them dying after the development has reached the stage in which the remant of the yolk-sac is in the normal embryo converted into the anterior body wall. As far as could be determined from the embryos in toto it is the enormously large (œdematous?) pericardia that mechanically obstruct the formation of the ventral body wall. The correctness of this interpretation will be tested by microscopic sections of these embryos.

The mechanism of the formation of the described monsters can at this time not even be definitely suggested. The observation was made that the yolk-sac in all extremely malformed embryos shows a marked decrease in size as compared with that of normal eggs of the corresponding stage of development. The greater the degree of injury inflicted on the embryo the smaller the yolk-sac. It is not impossible that the chemicals used in these experiments indirectly bring about this decrease in the size of the yolk-sac. For it was noticed that the chemicals used in these experiments softened the egg-membrane considerably, a fact which suggests an increase in the permeability of the egg. Owing to both increased permeability of the germ-disc cells and to internal osmotic pressure of the yolk-sac, an escape of substance from the yolk-sac might have been caused, which, being forced out at different points of the yolk-sac, might have fragmented the germ-disc. Many eggs were observed in which this fragmentation of the germ-disc was very evident. Some parts of this ruptured germ-disc may be so badly damaged as not to be able to develop further, while the remaining fragments, even if they are very small, may still give rise to various monsters, hemiembryos, dwarfs or even to a solitary eye. Or possibly the decrease in size of the yolk-sac of malformed ova may point to

elimination of both yolk-sac and germ substance as an effect of the solvent action of the chemicals to which the eggs were exposed. Whatever the mechanism involved in the production of the recorded pathological ova may be, at the present time, it can hardly be more than conjectured. It will be the object of future investigations to find a satisfactory answer to this open question.

There seems to be a close similarity between these cases where parts of the germ-disc are apparently lost through elimination of some kind and the production of hemiembryos by mechanically injuring one of the blastomeres of the developing frog's egg, as described by Roux, Morgan and other investigators.

The writer intends to continue this work on the teleost eggs as well as on the amphibian and hen's eggs. He also hopes that he may in the near future secure adequate facilities for carrying on experiments on the influence of the toxic substances of pathological metabolism on the development of the mammalian embryo. The plan of this work would be to mate animals in which metabolic disturbances had been produced experimentally.

A complete description and analysis of the results obtained in the investigation reported here will be published at an early date.

The writer takes pleasure in acknowledging his indebtedness to Professor C. R. Stockard of Cornell University Medical College with whom he on several occasions had discussed some phases of the work, and from whom he has received valuable suggestions regarding preservation of material.

MARINE BIOLOGICAL LABORATORY,
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NOTICE TO CONTRIBUTORS

Beginning with Vol. 29, No. 1, July, 1915, the Biological Bulletin will accept for publication papers in the field of Botany of general biological interest, such as heredity, cytology and physiology. It will thus more completely serve the interests of the Marine Biological Laboratory. Papers are limited to 25 pages and the cost of illustration in excess of $10.00 for any paper is chargeable to the author. One hundred reprints without covers are furnished free to contributors.

Dr. George T. Moore, The Missouri Botanical Garden, St. Louis, Mo., has consented to serve on the editorial board in the interest of botanical contributors, and manuscripts should be sent to his address for examination.

FRANK R. LILLIE,
Managing Editor.